Detection and Discrimination of *Pratylenchus neglectus* and *P. thornei* in DNA Extracts from Soil

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ABSTRACT

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A species-specific polymerase chain reaction (PCR) method was developed to detect and identify the root-lesion nematodes *Pratylenchus neglectus* and *P. thornei* from soil. A primer set was designed from *Pratylenchus* 28S rRNA gene sequences of the D3 expansion domain. Primer specificity was confirmed with 23 isolates of 15 nematode species and other plant-parasitic and non-plant-parasitic nematodes typically present in the soil communities, and with six fungal species commonly associated with wheat root rot. DNA obtained using a commercially available kit and a method developed in our laboratory gave comparable amplification. PCR conditions were optimized and the two species were differentiated by PCR products of 144 bp for *P. neglectus* and 288 bp for *P. thornei*. With this assay, we detected a single juvenile in 1 g of sterile, inoculated soil. Examination of 30 field soil samples revealed that this method was applicable to a range of soils naturally infested with these two pathogens in Oregon. This PCR-based method is rapid, efficient, and reliable, does not require expertise in nematode taxonomy and morphology, and could be used as a rapid diagnostic tool for commercial and research applications for disease forecasting and management.

Additional keywords: detection sensitivity, DNA extraction, DNA purification

Root-lesion nematodes, *Pratylenchus* spp., are widely distributed and economically important migratory endoparasites of many plant species (8). Recently, *Pratylenchus neglectus* and *P. thornei* were shown to be widespread in wheat fields in Oregon and Washington (27), and Idaho (31). High populations of these species in commercial fields have reduced yields of intolerant wheat cultivars by as much as 60% (29,30). *Pratylenchus* spp. populations exceeding the threshold for economic damage are now thought to occur in up to 60% of wheat fields in the Pacific Northwest. *P. neglectus* is more widely distributed than *P. thornei*, but mixed populations have been found within the same field (27).

The best approach to control damage from lesion nematodes is to grow cultivars that are both resistant and tolerant (28,34,36). Individual wheat cultivars differ in tolerance to these nematodes; cultivars that exhibit tolerance to *P. neglectus* are not necessarily tolerant to *P. thornei*, and vice versa. Mechanisms for resistance to these species are also under different species-specific genetic controls (36). Therefore, optimal cultivar selection requires that the lesion nematode species present in each field or region be accurately identified. Identification of these species will become even more important if current biofuels initiatives result in additional production of biofuel crops. For example, increasing canola acreage has the potential to significantly affect the level of damage to subsequent wheat crops, depending on the *Pratylenchus* spp. present. Canola may increase populations of *P. neglectus* but is less likely to increase populations of *P. thornei* (36).

Distinction between *P. neglectus* and *P. thornei* based on morphological characteristics requires detailed microscopic measurements by an experienced nematologist. Morphological diagnosis may be further complicated by difficulties in distinguishing three key diagnostic features (lip annules, tail shape, and vulva position). For example, vulval position has an overlapping range for these species, requiring measurements of multiple specimens to determine a mean for the ratio of vulval position to body length (12,13). In addition, morphological identification is limited to examination of mature adult females of these parthenogenic species. The largest commercial nematode diagnostic laboratory in the Pacific Northwest quantifies *Pratylenchus* spp. at the genus but not species level due to issues relating to reliability and expense. However, the lab is equipped and staffed for PCR diagnostic services using plant and soil DNA. A quick, reliable, and inexpensive molecular method that is amenable to high-throughput diagnostic labs is needed for distinguishing *P. neglectus* and *P. thornei*. A commercial testing program in South Australia provides DNA-based diagnostic services; however, the protocols are currently proprietary (21). A publically available procedure is needed to increase the level of service and diagnostic efficiency in laboratories elsewhere in the world, and particularly to facilitate more precise surveys of species distribution in the Pacific Northwest where *P. neglectus* and *P. thornei* are widespread and damaging.

Polymerase chain reaction (PCR)-based techniques have been reported for identifying species of *Pratylenchus* (22,32,37). The combination of PCR and restriction fragment-length polymorphism (RFLP) has been used to discriminate *Pratylenchus* spp. (32,37), but the RFLP portion of the test requires an additional step and time that is not amenable to commercial, high-throughput applications. PCR amplification with species-specific primers followed by gel electrophoresis has been used effectively for discrimination of some plant-parasitic nematodes (1,3,4,6,18,35,40). Al-Banna et al. (1) distinguished *P. neglectus* and *P. thornei* along with four other *Pratylenchus* spp. using PCR and species-specific primers derived from the internal variable portion of the D3 expansion region of the 28S rDNA. Carrasco-

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Ballesteros et al. (6) identified *P. thornei* from different life stages of the nematode using PCR and specific sequence-characterized amplified region (SCAR) primers derived from a randomly amplified DNA fragment.

Although the PCR-based methods described above distinguished *P. neglectus* from *P. thornei*, they were designed to use DNA extracted from nematode cultures or isolated individuals. There is a clear need for quick diagnostic methods that do not require time-consuming nematode extraction (38), such as are currently available for detecting plant-pathogenic bacteria and fungi in soil (9,11,15). There are few reports on the use of molecular techniques for detecting and distinguishing nematodes species in soil. Atkins et al. (4) detected false root-knot nematodes (*Nacobbus* spp.) from soil and potato tubers using PCR with species-specific primers and DNA extracted with a soil DNA extraction kit. Iwahori et al. (14) detected root-knot nematodes (*Meloidogyne* spp.) from soil using PCR-RFLP with DNA isolated with a modified commercial soil DNA kit.

The objectives of this study were to develop a quick, sensitive, reliable, and nonproprietary diagnostic method for identifying *P. neglectus* and *P. thornei* directly from soil, and to determine whether the method was applicable to a wide range of soils inhabited by these nematodes at a range of population densities.

MATERIALS AND METHODS

DNA extraction from nematodes. Nematodes were extracted from soil samples using the Whitehead tray method (39). This method relies principally on the active movement of migratory nematodes from the moist soil sample into the surrounding water. Adult females were morphologically identified (12,13) as either *P. neglectus* (two lip annules, pointed but still round tail, and vulva position ratio on body at 76 to 87%) or *P. thornei* (three lip annules, slightly truncated tail on end, and vulva ratio at 73 to 80%), and then placed onto surface-sterilized carrot disks to establish pure cultures. DNA was extracted from nematodes following the protocol described by Waeyenberge et al. (37), with some modifications. Ten nematodes of mixed juvenile and adult stages were handpicked using a dental pick, put into 20 µl of sterilized nanopure water on a concave glass slide, and cut into two pieces under a dissecting microscope. Then, 10 µl of suspension containing nematode pieces were pipetted into a 0.2-ml sterile Eppendorf tube with 8 µl of lysis buffer (500 mM KCl; 100 mM Tris-Cl, pH 8.3; 15 mM $MgCl_2$; 10 mM dithiothreitol [DTT]; 4.5% Tween 20; and 0.1% gelatin). The tube contents were frozen at –20°C for at least 20 min, then thawed, and 2 µl of proteinase K at 600 µg/ml was added. The tubes were incubated at 65°C in a water bath for 1 h, and consecutively at 95°C for 10 min to inactivate proteinase K. The nematode lysis mix was centrifuged at 16,000 × *g* for 5 min and the supernatant was transferred to a new 0.2-ml tube and stored in –20°C until used as the DNA template.

Primer selection and evaluation for specificity. The species-specific forward primer PTHO and the common reverse primer D3B described by Al-Banna et al. (1) were used to identify *P. thornei* (Table 1). The species-specific forward primer PNEG-F1 and the common reverse primer D3B5 were designed to identify *P. neglectus*. PNEG-F1 was designed based on the variable region in the alignment of the 28S rRNA D3 expansion domains obtained from GenBank. D3B5 was selected from the conserved region of the same D3 expansion domain in order to produce a PCR fragment different in size from that produced with DNA from *P. thornei*. These primers were analyzed for annealing temperature, GC content, dimers, and hairpin loops using GeneRunner (version 3.05; Hastings Software, Inc., Hudson, NY), and synthesized by Invitrogen (Carlsbad, CA).

Six isolates of *P. neglectus* and two isolates of *P. thornei* from Oregon, Washington, Montana, and Idaho (Table 2) were used to examine the specificity of the *Pratylenchus* primers. Isolates of five other *Pratylenchus* spp. and three *Meloidogyne* spp. (Table 2) were tested for primer specificity. Five plant-parasitic nematode species (*Heterodera avenae*, *H. filipjevi*, *Tylenchorhynchus* sp., *Merlinius brevidens*, and *Paratylenchus* sp.), three nematode communities frequently found in wheat fields in eastern Oregon, and six fungal species commonly associated with wheat root diseases were also used as controls (Table 2). PCR reactions of 25 µl contained the DNA template (5 µl), 0.75 units of *Taq* polymerase (Roche, Mannheim, Germany), 200 µM dNTPs, 0.5 µM each primer, 1× PCR buffer with 1.5 mM $MgCl_2$, and 1× Cresol Red in 20% glycerol. PCR amplification was performed in a MyCycler Thermal Cycler (Bio-Rad, Richmond, CA) as follows: 95°C for 3 min followed by 35 cycles of 95°C for 30 s, 60°C (PNEG-F1/D3B5) or 62°C (PTHO/D3B) for 30 s, and 72°C for 30 s, with a final extension at 72°C for 7 min. PCR products were separated in 2% standard agarose gels stained with ethidium bromide. Molecular size was estimated by a 100-bp DNA ladder (Roche). Band pattern was photographed under UV light using a Polaroid digital camera, and analyzed by the Polaroid PhotoMAX Pro program (Polaroid, Bedford, MA). This experiment was performed four times.

To expand specificity evaluation to additional nontarget nematode species that could not be obtained in culture or DNA based on requests, an in silico analysis was conducted using GenBank D3 expansion sequences from four *Pratylenchus* spp. and four nematode genera and the computer software PrimerSelect 5.00 (DNASTAR, Inc, Madison, WI). Primer specificity was determined by the primer-template duplex stability values (ΔG) as described by Schroeder et al. (26) and Okubara et al. (20). The *Pratylenchus* spp. were *P. vulnus* (U47547 and AJ545020), *P. hexincisus* (U47554 and AF303949), *P. brachyurus* (U47553), and *P. coffeae* (U47552, AF170428, and AF170436). The nematode species in other genera were *Meloidogyne incognita* (AY355417), *Globodera tabacum solanacearum* (AF393846), *G. pallida* (AF393843), *Paratrichodorus pachydermus* (AM180727), *P. anemones* (AJ781505), and *H. glycines* (DQ328692).

DNA extraction from soil and DNA purification by polyvinylpolypyrrolidone powder column. Various extraction methods were evaluated by examining and comparing band intensity of PCR amplification on agarose gel. Total genomic DNA was extracted from soil using two commercial soil DNA extraction kits, FastDNA SPIN Kit for Soil (Bio101, La Jolla, CA) and PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA), according to the manufacturers' recommendations. Four buffers prepared in the laboratory also were compared. These were designated buffer A (pH 8.0, 30 mM Na_2HPO_4 and 90 mM NaH_2PO_4) (38), buffer B (200 mM Tris-HCl, pH 8.5; 250 mM NaCl; 25 mM EDTA; 0.5% sodium dodecyl sulfate [SDS]) (25), buffer C (500 mM KCl; 100 mM Tris-Cl, PH 8.3; 15 mM $MgCl_2$; 10 mM DTT; 4.5% Tween 20; 0.1% gelatin) (37), and buffer D (equal amounts of

Table 1. Sequences of polymerase chain reaction (PCR) primers and the expected size of PCR products to discriminate *Pratylenchus neglectus* and *P. thornei*

Species	Primer name[a]	Sequence (5′–3′)	Band size (bp)	Reference
P. neglectus	F: PNEG-F1	CGCAATGAAAGTGAACAATGTC	144	This study
	R: D3B5	AGTTCACCATCTTTCGGGTC	…	This study
P. thornei	F: PTHO	GAAAGTGAAGGTATCCCTCG	288	1
	R: D3B	TCGGAAGGAACCAGCTACTA	…	1,2,7

[a] F = forward primer and R = reverse primer.

phosphate buffer [100 mM NaH_2PO_4, pH 8.0] and SDS buffer [100 mM NaCl; 500 mM Tris, pH 8.0; 10% SDS]) (11). DNA extractions with these buffers were performed after mechanical lysis using lysing matrix tubes provided in the FastDNA SPIN Kit, processed in a FastPrep FP120 machine (Bio101, Savant) for 30 s at a speed of 5.5 m/s. Crude DNA extracts were then purified using High Pure spin filters (Roche) that contained water-insoluble polyvinylpolypyrrolidone (PVPP; Sigma-Aldrich, St. Louis) powder.

To replace the expensive lysing matrix provided in FastDNA SPIN Kit, the effect of glass beads was assessed, including 0.20 g of 1.0-mm glass beads (Research Product International Corp., Mt. Prospect, IL) with one ceramic sphere (6.4 mm; Fisher Scientific, Pittsburgh), 0.80 g of 1.0-mm glass beads, 0.53 g of 1.0-mm glass beads, and 0.60 g of 1.0-mm glass beads combined with 0.15 g of 0.1-mm glass beads.

Extraction of DNA from soil often results in co-extraction of humic substances that inhibit DNA polymerase during PCR amplification. To remove humic substances prior to PCR reaction, PVPP columns were prepared as a substitute for spin filters provided in the extraction kits. A hole (1 mm) was made in the bottom of a 0.5-ml tube and was covered by small pieces (0.005 g) of glass wool (Corning, NY) using a forceps. The tube was inserted into a 1.5-ml tube with the lid removed. Dry PVPP powder (0.05 g; Sigma-Aldrich) was added to the 0.5-ml tube. The PVPP column was conditioned by the addition of 100 µl of sterilized nanopure water ($SNPH_2O$) twice, each followed by 3 min of centrifugation at 400 × *g*. A final spin for 30 s was performed just before use to remove residual water.

The optimized protocol developed in this study is as follows. Soil was added to a 2.0-ml screw-cap tube (Bio-Rad) with glass beads (0.53 g, 1 mm). Buffer D (300 µl of each phosphate buffer and SDS buffer) was added and the tube was inverted several times. Chloroform:isoamyl alcohol (400 µl, 24:1) was added to the tube and shaken in a FastPrep FP120 machine for 30 s at a speed of 5.5 m/s. The tube was centrifuged for 5 min at 16,000 × *g* and the supernatant (less than 600 µl) transferred to a clean 1.5-ml centrifuge tube. Then, 300 µl of cold NaOAc (3 M, pH 5.2) was added and, following incubation (5 min at –20°C), the suspension was cleared by centrifugation for 5 min at 16,000 × *g*. The supernatant (up to 650 µl) was transferred to a clean tube. DNA was subsequently precipitated with 600 µl of cold isopropanol for 20 min at room temperature and collected by centrifugation at 16,000 × *g* for 5 min. The resulting pellet was rinsed with 70% ethanol and centrifuged for 5 min at 16,000 × *g*. The DNA pellet was air dried and dissolved in 100 µl of $SNPH_2O$. The crude DNA solution was added to the PVPP column described above, followed by centrifugation at 400 × *g* for 1 min, incubation at room temperature for 2 min, and a final centrifugation at 400 × *g* for 5 min. The purified DNA in the tube was then used for PCR.

Optimization of PCR conditions with DNA extracted from soil. To improve PCR amplification for *P. neglectus*, the effect of bovine serum albumin (BSA) as a PCR enhancer was assessed. A BSA solution of 10 µg/µl was added to PCR reaction mixtures to make final concentrations of 0, 0.1, 0.4, 0.8, and 1.0 µg/µl. Additional

Table 2. Isolates of *Pratylenchus* spp. and other nematode and fungal species used to examine the species-specific polymerase chain reaction primers for distinguishing *P. neglectus* and *P. thornei*

Species[a]	N or F[b]	Isolate	Origin	Host	Source[c]
Pratylenchus neglectus	N	Pn1	La Grande, OR	Wheat	R. Smiley
P. neglectus	N	Pn2	Lind, WA	Wheat	R. Smiley
P. neglectus	N	Pn3	Moro, OR	Wheat	R. Smiley
P. neglectus	N	Pn4	Heppner, OR	Wheat	R. Smiley
P. neglectus	N	Pn5	Great Falls, MT	Wheat	A. Dyer
P. neglectus	N	Pn6	Canyon County, ID	Potato	S. Hafez
P. thornei	N	Pt1	Pendleton, OR	Wheat	R. Smiley
P. thornei	N	Pt2	Pendleton, OR	Wheat	R. Smiley
P. agilis	N	031302	Wye, MD	Corn	L. Carta
P. crenatus	N	012204	Clarksville, MD	Grass	Z. Handoo
P. zeae	N	030204	North Carolina	Corn	L. Carta
P. scribneri	N	062805	Homestead, FL	Tomato	W. Klassen
P. scribneri	N	032102	Seneca County, OH	Corn	L. Carta
P. penetrans	N	030402	New York	Corn	L. Carta
P. penetrans	N	052704	Wisconsin	Potato	A. MacGuidwin
Meloidogyne naasi	N	110704	Linn County, OR	Oat, wheat	K. Merrifield
M. chitwoodi	N	110504	Parma, ID	Potato	S. Hafez
M. hapla	N	070808	Prosser, WA	Grape	E. Riga
Heterodera avenae	N	Ha	La Grande, OR	Wheat	R. Smiley
H. filipjevi	N	Hf	La Grande, OR	Wheat	R. Smiley
Tylenchorhynchus sp.	N	Ty	Pendleton, OR	Wheat	S. Easley
Merlinius brevidens	N	Mb	Pendleton, OR	Wheat	A. Thompson
Paratylenchus sp.	N	Pa	La Grande, OR	Wheat	G. Yan
Nematode community 1	N	Nc1	Pendleton, OR	Wheat	J. Sheedy
Nematode community 2	N	Nc2	Pendleton, OR	Wheat	J. Sheedy
Nematode community 3	N	Nc3	Pendleton, OR	Wheat	J. Sheedy
Bipolaris sorokiniana	F	103-18	Walla Walla, WA	Wheat	R. Smiley
Fusarium culmorum	F	R5321	Chatham, Ontario, Canada	Wheat	R. Smiley
F. pseudograminearum	F	032-06	Moro, OR	Wheat	R. Smiley
Gaeumannomyces graminis var. *tritici*	F	99401	Dayton, WA	Wheat	R. Smiley
Rhizoctonia oryzae	F	2-3-2	Wenatchee, WA	Wheat	M. Mazzola
R. solani AG-8	F	1727B	Wenatchee, WA	Wheat	M. Mazzola

[a] Nematode communities 1, 2, and 3 were extracted from soils that were not infested with any *P. neglectus* or *P. thornei* but were infested with many other plant-parasitic and non-plant-parasitic nematodes; the Whitehead tray method was used for nematode extraction.

[b] N = nematodes and F = fungi.

[c] Isolates were obtained from R. Smiley, S. Easley, G. Yan, A. Thompson, and J. Sheedy, Oregon State University, Columbia Basin Agricultural Research Center, Pendleton; A. Dyer, Montana State University, Bozeman; S. Hafez, University of Idaho, Southwest Idaho Research and Extension Center, Parma; L. Carta and Z. Handoo, United States Department of Agriculture-Agricultural Research Service (USDA-ARS), Nematology Laboratory, Beltsville, MD; W. Klassen, University of Florida, Tropical Research and Education Center, Homestead; A. MacGuidwin, University of Wisconsin, Department of Plant Pathology, Madison; K. Merrifield, Oregon State University, Department of Botany and Plant Pathology, Corvallis; E. Riga, Washington State University, Irrigated Agriculture Research and Extension Center, Prosser; and M. Mazzola, USDA-ARS, Wenatchee, WA.

adjustments made to the previously described PCR protocol included decreasing the DNA template from 5 to 1 µl for *P. neglectus* and from 5 to 0.2 µl for *P. thornei*. The number of PCR cycles was increased from 35 to 40 for both species.

Detection sensitivity of PCR amplification. Soil was inoculated with *P. neglectus* or *P. thornei* to evaluate the sensitivity of PCR detection. A Walla Walla silt loam that was not infested with *P. neglectus* and *P. thornei* was collected from Pendleton, OR and was sieved through a 3-mm sieve. Thereafter, the soil was autoclaved (121°C, 115 kPa) two times for 45 min each to completely kill all living organisms. Juveniles of *P. neglectus* and *P. thornei* were added separately to 1 g of autoclaved soil using a dental pick with the aid of a microscope. Concentrations of 0, 1, 2, 3, and 5 juveniles/g of soil were used. Nematode DNA was extracted from the soils using the method developed in this study and DNA was quantified using the NanoDrop ND-1000 Spectrophotometer (Wilmington, DE). The DNA extraction procedure was conducted from eight different samples for each level of inoculation. PCR reactions were performed in duplicate for each independent DNA extraction under the optimum conditions for the soil samples as described above with BSA at a final concentration of 0.8 µg/µl. The sensitivity for PCR amplification was determined by the ability to detect a minimum number of juveniles/g of soil.

Validation of species identification in soil. Sixteen soil samples were collected from various fields between Heppner and Condon in Morrow County, OR during 2006 and 2007. This location was known to be significantly infested with *P. neglectus* and minimally infested with *P. thornei*. Fourteen soil samples were taken from fields adjacent to the Columbia Basin Agricultural Research Center near Pendleton in Umatilla County, OR during 2006 and 2007. This location was known to be highly infested with *P. thornei* but minimally infested with *P. neglectus*. Nematodes were isolated from approximately 200 g of fresh moist soil using the Whitehead tray method. Nematodes in 1 ml of extracted suspension were identified and quantified on a nematode-counting slide under a microscope and converted to the number per kg of soil. Nematodes were identified as *P. neglectus* and *P. thornei* (13), other plant-parasitic nematode genera (17), and non-plant-parasitic nematodes; the numbers for each group are listed in Table 3. DNA was extracted from 0.5 g of moist soil using both the PowerSoil DNA Isolation Kit and our in-house method. DNA extractions were conducted four times for each sample and PCR was performed under the optimum conditions for soil samples, as described above. The identifications determined by PCR assay were compared with morphological identifications. A no-DNA template was used as a negative control and DNA from pure nematode cultures was used as positive controls.

RESULTS

Primer specificity. The primer PNEG-F1 designed for *P. neglectus* amplified a unique PCR product (144 bp) with DNA from *P. neglectus* isolates Pn1, Pn2, Pn3, Pn4, Pn5, and Pn6 when combined with primer D3B5 but did not produce an amplicon with DNA from *P. thornei* isolates Pt1 and Pt2 (Fig. 1A). As expected, the primer pair PTHO/D3B amplified a specific band (288 bp) from *P. thornei* cultures Pt1 and Pt2 but did not generate an amplicon with DNA from *P. neglectus* cultures Pn1, Pn2, Pn3, Pn4, Pn5, and Pn6

Table 3. Numbers of *Pratylenchus neglectus*, *P. thornei*, and other plant-parasitic and non-plant-parasitic nematodes extracted from naturally infested soils using the Whitehead tray and morphological identification procedures and polymerase chain reaction (PCR) assays

		Nematodes/kg of soil[a]				PCR assay[b]	
Soil[c]	Year collected	Pn	Pt	OP	NP	Pn	Pt
S1	2007	87	0	1,136	3,843	–	–
S2	2007	93	0	0	3,988	–	–
S3	2007	113	0	0	10,251	–	–
S4	2007	343	0	2,286	5,373	+	–
S5	2007	815	0	0	4,158	+	–
S6	2007	1,237	0	0	4,948	+	–
S7	2007	2,697	0	0	4,238	+	–
S8	2007	3,609	570	190	30,388	+	+
S9	2006	4,185	0	135	3,240	+	–
S10	2007	4,689	0	408	2,854	+	–
S11	2006	6,740	0	124	3,463	+	–
S12	2007	7,774	0	2,418	11,056	+	–
S13	2007	11,118	0	139	2,085	+	–
S14	2007	13,704	98	196	25,059	+	–
S15	2007	16,836	324	324	8,256	+	+
S16	2007	17,959	0	770	12,828	+	–
S17	2007	0	0	2,575	3,277	–	–
S18	2007	0	0	1,033	2,195	–	–
S19	2007	0	0	898	2,437	–	–
S20	2007	0	117	0	1,756	–	–
S21	2007	0	126	631	1,767	–	+
S22	2006	0	315	473	630	–	+
S23	2007	0	635	741	4,340	–	+
S24	2007	0	1,936	129	1,678	–	+
S25	2007	0	3,277	0	2,622	–	+
S26	2007	0	4,903	0	2,790	–	+
S27	2007	0	7,198	0	1,400	–	+
S28	2007	0	9,430	0	982	–	+
S29	2006	0	13,721	0	1,083	–	+
S30	2007	0	15,998	0	4,081	–	+

[a] Pn = *P. neglectus*, Pt = *P. thornei*, OP = other plant-parasitic nematodes, and NP = non-plant-parasitic nematodes.

[b] Presence (+) or absence (–) of Pn (144 bp) or Pt (288 bp) were detected by PCR using PNEG-F1/D3B5 or PTHO/D3B, respectively.

[c] Soils were collected in Morrow (S1-S16) and Umatilla (S17-S30) Counties of Oregon.

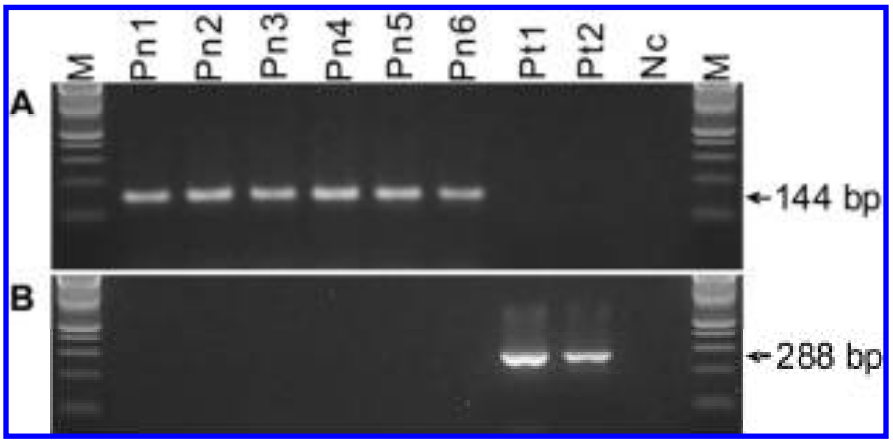

Fig. 1. Specific polymerase chain reaction amplification for *Pratylenchus neglectus* (144 bp) and *P. thornei* (288 bp) from pure cultures using species-specific primers. **A,** Amplified with *P. neglectus*-specific primer set PNEG-F1/D3B5. **B,** Amplified with *P. thornei*-specific primer pair PTHO/D3B. DNA templates from the isolates Pn1, Pn2, Pn3, Pn4, Pn5, Pn6, Pt1, and Pt2 (Table 2) were used. Nc: negative control without DNA template; M: 100-bp DNA molecular weight ladder.

(Fig. 1B). The sizes of the specific PCR fragments were consistent with those inferred from the nucleotide sequences. Furthermore, no specific PCR product was observed when tested with DNA extracted from five other nontarget *Pratylenchus* spp. (a total of seven isolates) and three *Meloidogyne* spp. (Table 2). Similarly, no specific PCR product was produced with DNA from five other plant-parasitic nematode species (*H. avenae*, *H. filipjevi*, *Tylenchorhynchus* sp., *Merlinius brevidens*, and *Paratylenchus* sp.), three nematode communities extracted from local wheat field soils, and six fungal species (Table 2). In silico analysis with four other related *Pratylenchus* spp. and six species in four nematode genera showed that the new species-specific primer PNEG-F1 does not form hybrids with the sequences of these nontarget organisms because the stability values (ΔG) obtained for these nontarget hybrids were below the default stability cut-off (–33 kcal/mol).

DNA extraction from soil. Both commercial kits and all extraction buffers tested in this study allowed the detection of *P. thornei* and *P. neglectus* in soil (Fig. 2). MoBio PowerSoil DNA Kit (lane 3) generated a slightly brighter PCR band than that by FastDNA SPIN Kit (lane 4). Among the four buffers, buffer D (lane 8) produced the brightest amplification band. Buffer C that was used for DNA extraction from nematode cultures (lane 7) was least effective, with relatively faint amplification (Fig. 2A). There was no difference in the intensity of bands observed when using the buffer A (lane 5) and buffer B (lane 6). Therefore, we used buffer D in further experiments. Notably, *P. thornei* DNA extracted with buffer D (lane 8) produced an amplification fragment as bright as that with the MoBio PowerSoil DNA Kit (Fig. 2A, lane 3). Similarly, *P. neglectus* DNA extracted from soil with buffer D (lane 8) also produced a band almost as bright as that with the MoBio PowerSoil DNA Kit (Fig. 2B, lane 3).

All four treatments using glass beads yielded DNA and allowed the detection of *P. thornei* with the FastPrep FP120 instrument and buffer D. The brightest bands were achieved with 0.53 g of 1.0-mm glass beads as well as 0.20 g of 1.0-mm glass beads combined with one ceramic sphere. Minimal amplification resulted from 0.60 g of 1-mm glass beads combined with 0.15 g of 0.1-mm glass beads. Intermediate amplification was obtained with 0.80 g of 1.0-mm glass beads. A similar effect was observed for detection of *P. neglectus* from soil.

Removal of PCR inhibitors in soil. No PCR amplification product was produced with crude DNA extracts obtained with each of the four extraction buffers. To rule out PCR inhibitors co-extracted from soil, PVPP columns were used to replace spin filters provided in commercial kits. After centrifugation at 400 × *g*, DNA extracts from all samples produced visible amplicons without further dilution of the DNA extracts. Higher speed (720 × *g*) centrifugation for 5 min resulted in DNA extracts that could not be amplified with the same PCR conditions.

PCR amplification of nematode DNA from soil. In spite of the special PVPP purification step, PCR amplification from soil extracts with the *P. neglectus*-specific primer set was relatively weak compared with that from nematode pure cultures. To improve the amplification, BSA was added to the PCR reaction mixtures at different concentrations (0.1, 0.4, 0.8, and 1.0 µg/µl). No marked difference was observed for the band intensity between different concentrations of BSA; however, the presence of BSA enhanced PCR amplification over control samples. The effect of BSA on PCR using DNA extracted with our in-house method was more evident than on DNA extracted with the PowerSoil DNA Isolation Kit. The addition of BSA (0.8 µg/µl) also allowed amplification at a wider range of DNA concentrations, from 0.1 to 2.7 ng/µl. In contrast, detection of *P. thornei* from soil could be achieved without BSA. However, PCR amplification for *P. thornei* required a lower concentration of DNA from 0.1 to 0.5 ng/µl.

Detection sensitivity of the PCR reaction in artificially inoculated soils. Varying numbers of *P. neglectus* and *P. thornei* juveniles (n = 1, 2, 3, and 5) were inoculated into 1 g of sterile soils. Specific bands were amplified from all levels of the inoculation for both species even when one nematode was added (Fig. 3). No band was generated from the control soil (sterilized but not inoculated). The PCR test can detect one *P. neglectus* juvenile per gram of soil in seven of the eight independent DNA extractions and one *P. thornei* juvenile per gram of soil in 100% of the assays. This detection sensitivity equates to half the

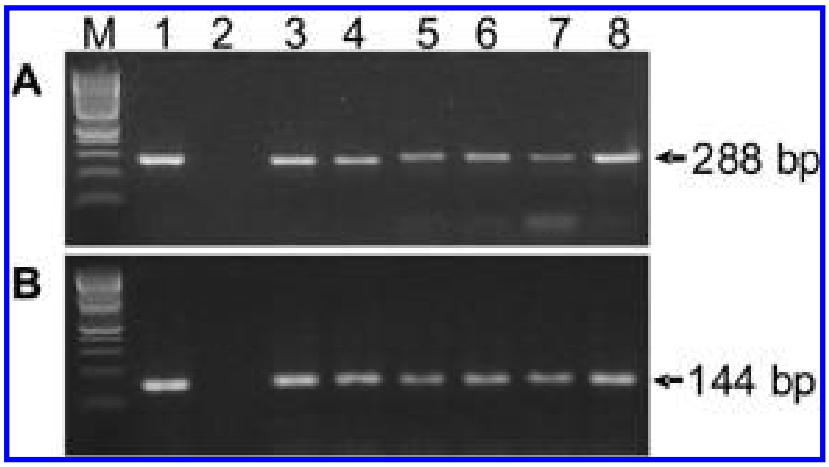

Fig. 2. Effect of DNA extraction methods on polymerase chain reaction (PCR) detection. **A,** DNA from the soil sample S29 was used for PCR amplification with *Pratylenchus thornei*-specific primer pair PTHO/D3B: lane 1, positive control using DNA from hand-picked *P. thornei*. **B,** DNA from the soil sample S11 was used for PCR amplification with *P. neglectus*-specific primer set PNEG-F1/D3B5: lane 1, positive control using DNA from hand-picked *P. neglectus*; lane 2, negative control without DNA template; lane 3, PowerSoil DNA Isolation Kit; lane 4, FastDNA SPIN Kit for Soil; lane 5, buffer A; lane 6, buffer B; lane 7, buffer C; lane 8, buffer D; lane M, 100-bp DNA molecular weight ladder.

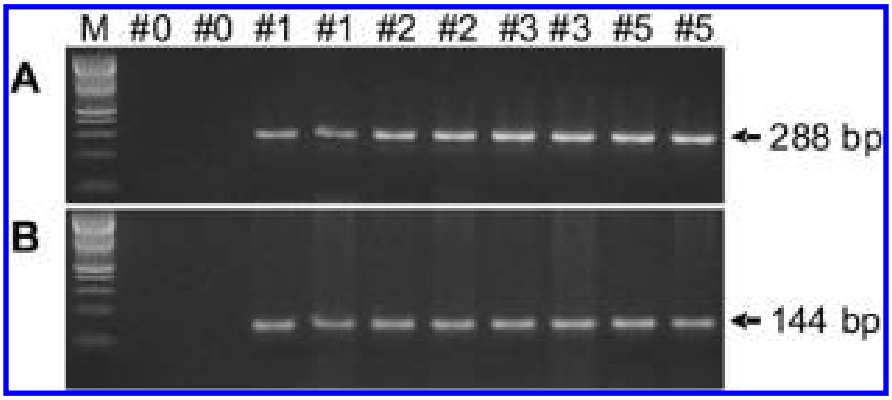

Fig. 3. Level of detection for the polymerase chain reaction amplification in artificially inoculated soils. **A,** Inoculation of *Pratylenchus thornei*: zero juvenile (J) (#0), one J (#1), two J (#2), three J (#3), and five J (#5); **B,** Inoculation of *P. neglectus*: zero J (#0), one J (#1), two J (#2), three J (#3), and five J (#5); M: 100-bp DNA ladder.

estimated economic threshold (2,000 nematodes/kg of soil) for natural infestations in dryland fields in the Pacific Northwest (30). The specific amplicons were produced in each of the eight replicates for the other inoculum levels.

Discrimination of *P. neglectus* and *P. thornei* in naturally infested soils. The developed protocol was validated for detecting and discriminating *P. neglectus* and *P. thornei* in 30 soil samples harboring a range of population densities, from 0 to 17,959 *P. neglectus*/kg of soil and 0 to 15,998 *P. thornei*/kg of soil. In all, 2 of 16 soil samples (Table 3, S4 and S5) from the Morrow County field sites produced bands with the same size as the *P. neglectus*-positive control (144 bp) in three of the four independent DNA extractions when tested with PNEG-F1/D3B5. Eleven samples (S6 to S16) produced the expected amplicons in each replicate with PNEG-F1/D3B5. The other three samples (S1 to S3) did not produce the expected amplicon in any of the replicates. The limit of PCR detection for *P. neglectus* was 343 nematodes/kg of soil, as determined by the Whitehead tray method. The specific amplicon was not produced in any of the four replicates with the *P. thornei*-specific primer set PTHO/D3B for all except two samples. The amplicons were evident for these two samples (S8 and S15), which also contained 570 and 324 *P. thornei*/kg of soil, respectively, as determined by the Whitehead tray method. DNA from 2 of 14 samples (Table 3, S21 and S22) collected from Umatilla County sites generated the expected *P. thornei* amplicon (288 bp) in three of the four replicates when amplified with PTHO/D3B. DNA from eight samples (S23 to S30) also generated the expected *P. thornei* amplicon in each replicate. Sample S20 did not produce the expected amplicon in any of the replicates. The limit of PCR detection for *P. thornei* was 126 nematodes/kg of soil. The specific amplicon was not observed for the samples from S20 to S30 in any of the replicates with PNEG-F1/D3B5. Soil samples (S17, S18, and S19) that had no *P. neglectus* and *P. thornei*, according to the Whitehead tray method, also did not produce the specific amplicons in each replicate when tested with both primer pairs, confirming the specificity of the primers even with the presence of DNA from other plant-parasitic and nonparasitic nematodes in the samples (Table 3). The banding patterns for DNA extractions from nine soil samples obtained by our in-house DNA extraction assay and tested with both species-specific PCR primer sets is shown in Figure 4A. A similar banding pattern was observed for these DNA extractions obtained by the PowerSoil DNA isolation kit (Fig. 4B).

DISCUSSION

Two plant-parasitic root-lesion nematodes, *P. neglectus* and *P. thornei*, were detected and discriminated in a variety of soil samples using a DNA extraction and PCR amplification method developed in this study. This approach utilized mechanical disruption of nematodes within the soil sample in the FastPrep homogenizer, extracted DNA from soil with or without the use of a commercial kit, and applied species-specific primers to detect multicopy DNA of the target *Pratylenchus* spp. The assays were sensitive, reliably detecting one juvenile of genomic DNA in 1 g of sterile, inoculated soil. The assays also proved highly specific when evaluated against template DNA from a range of nematode and fungal species that are common in wheat fields in the Pacific Northwest. Although PCR assays to discriminate *Pratylenchus* spp. have been previously reported (1,6,32,37), they were not evaluated for detecting *P. neglectus* and *P. thornei* directly from soil.

For *P. thornei*, the species-specific primer PTHO described by Al-Banna et al. (1) was used. This primer produced a specific amplicon with *P. thornei* DNA but did not amplify DNA from other closely related *Pratylenchus* spp., including *P. neglectus*, *P. brachyurus*, *P. penetrans*, *P. scribneri*, and *P. vulnus* (1). For *P. neglectus*, the species-specific primer PNEG-F1 was designed from the variable region of D3 expansion domain of the 28S rRNA. No cross reactivity was observed between the sequence of this primer and DNA from other closely related *Pratylenchus* spp. reported in the Pacific Northwest by in silico analysis. Moreover, when challenged with five other nontarget *Pratylenchus* spp., eight nematode species in other genera, three nematode communities, and six fungal species, the specific amplicons were not produced. DNA from three soil samples that did not contain *P. neglectus* and *P. thornei* but had other plant-parasitic and non-plant-parasitic nematodes typical of dryland wheat fields in eastern Oregon also did not yield the specific amplicons, demonstrating the specificity of the primers in the soil community complex. Further

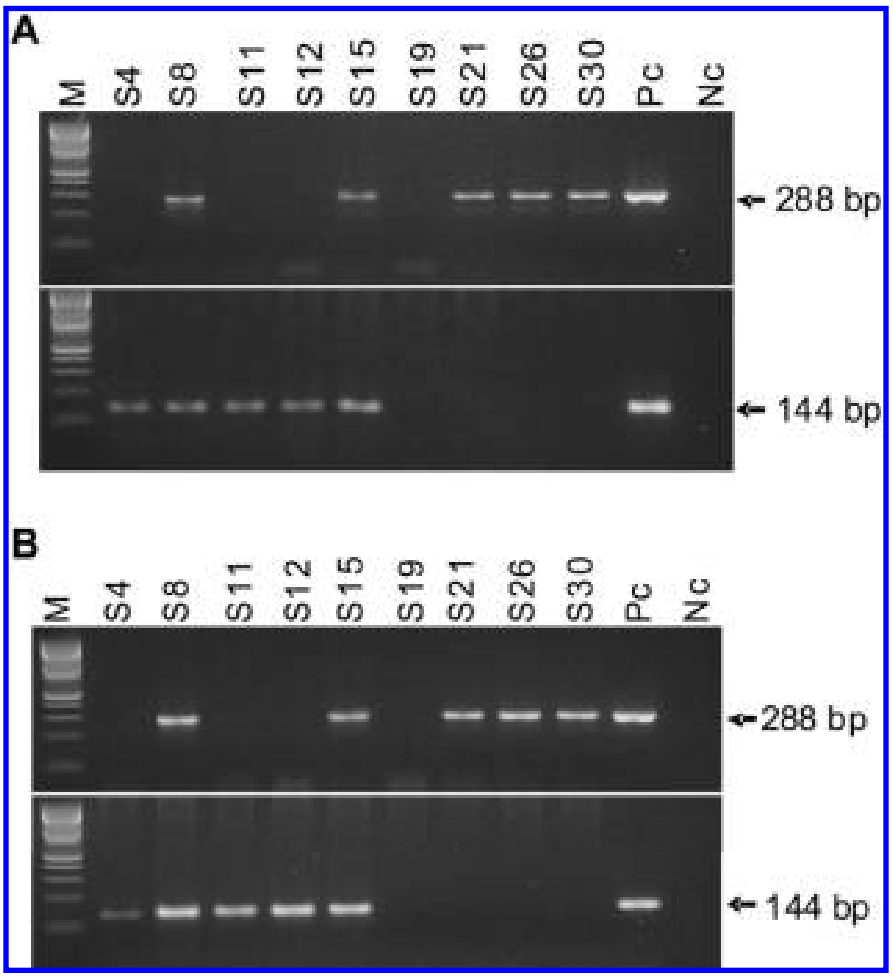

Fig. 4. Identification of *Pratylenchus thornei* and *P. neglectus* in naturally infested soil samples. **A,** Nematode DNA was extracted from soil without any components of a commercial kit. DNA was amplified with the *P. thornei*-specific primer set PTHO/D3B (top) and with the *P. neglectus*-specific primer set PNEG-F1/D3B5 (bottom). **B,** Nematode DNA was extracted from soil with the PowerSoil DNA Isolation Kit. DNA was amplified with the *P. thornei*-specific primer set PTHO/D3B (top) and with the *P. neglectus*-specific primer set PNEG-F1/D3B5 (bottom). The soil samples S4, S8, S11, S12, S15, S19, S21, S26, and S30 were shown in Table 3. Pc: positive control from nematode culture; Nc: negative control without DNA template.

testing of these primers with local species may be necessary if these primers were used for PCR identification with DNA extracts from soil in other areas.

Published species-specific primers (1) produced PCR fragments with very little difference (2 bp) in length for the two *Pratylenchus* spp. due to the use of a common reverse primer, D3B. Therefore, we designed a reverse primer D3B5 from the conserved sequences of 28S rRNA D3 expansion region. This resulted in PCR fragments that had larger size differences that could be easily separated by agarose gel electrophoresis. Additionally, the forward primer PNEG-F1 for *P. neglectus* proved to be more sensitive and robust for detection of DNA from soil extracts than the PNEG primer described by Al-Banna et al. (1) at the annealing temperature of 60°C.

The DNA extraction method developed in this study was based on the method of Damm and Fourie (11), which was developed to extract DNA from the soilborne fungal pathogens *Phaeomoniella chlamydospora* and *Cylindrocarpon* spp. With some modifications, we used this protocol to extract *Pratylenchus neglectus* and *P. thornei* DNA from soil for PCR detection. Adding 0.53 g of 1.0-mm glass beads or 0.20 g of 1.0-mm glass beads combined with one ceramic sphere to the extraction buffer increased the amplification intensity. Considering the relative cost of a ceramic sphere, 0.53 g of 1.0-mm glass beads were selected in place of the lysing matrix provided in FastDNA SPIN Kit. Glass beads aided in the disruption or shearing of the nematode cuticle, allowing release of DNA. Cullen and Hirsch (9) reported that the use of bead-beating in the cell lysis step increased the DNA yield and also decreased the amount of humic compounds when monitoring a *Rhizobium leguminosarum* bv. *viciae* strain RSM2004 in Rothamsted field soils. Kageyama et al. (15) reported that adding 0.2 g of 1-mm glass beads to the extraction buffer was necessary for detecting *Verticillium dahliae* in soil.

Purification of DNA from soil was essential for detecting the two target nematode species. Two merits of the DNA purification method developed in this study are that (i) it does not require the expensive Micro Bio-Spin column or Sephadex G-75 used by Cullen et al. (10) and (ii) it is easy to prepare the PVPP column. Extraction of DNA from soil frequently results in co-extraction of humic substances that interfere with PCR amplification (9,11,33). PVPP is an inexpensive chemical that can be utilized for eliminating the majority of humic and fulvic acids because it binds phenolic compounds (9–11). Commercial soil DNA extraction kits are expensive (e.g., the cost of the MOBio PowerSoil DNA kit is approximately $4.16 per reaction). Our in-house assay, using buffer D (phosphate and SDS buffer), 0.53 g of 1.0-mm disruption glass beads, glass wool, and purification column with dry PVPP powder yielded PCR-quality DNA that was comparable with that yielded by the PowerSoil DNA kit. The estimated cost for one reaction is $0.30, which greatly reduces the cost of DNA extraction and purification from soil.

The effect of BSA on PCR amplification was different for the two target nematode species at lower DNA concentrations (0.1 to 0.5 ng/µl). BSA greatly enhanced detection of *P. neglectus* in soil, whereas *P. thornei* could be detected without the addition of BSA. The effect of BSA on PCR was not correlated with BSA concentrations. Similarly, Kageyama et al. (15) found that BSA was essential for detecting *V. dahliae* and enhanced detection of *Pythium ultimum* but was not necessary for detecting *Plasmodiophora brassicae* in soil. BSA is thought to stabilize *Taq* polymerase and neutralize inhibitory contaminants (11,16,19,24).

PCR detected one juvenile in 1 g of sterile, inoculated soil, equating to 1,000 juveniles/kg of soil. It could detect even lower densities of nematodes in naturally infested soil: 126 *Pratylenchus thornei* nematodes/kg of soil and 343 *P. neglectus* nematodes/kg of soil. The discrepancy between artificially inoculated soil and naturally infested soil may be due to differences in sample size and nematode counting methods used. One juvenile was the minimum number of nematodes that can be added into 1 g of sterile soil, whereas the number of target nematodes from natural soil was obtained using the Whitehead tray method by counting the number of nematodes per milliliter of suspension extracted from approximately 200 g of soil and converting to the number in 1 kg of soil. More importantly, the detection sensitivity was much lower than the economic threshold level (2,000 nematodes/kg of soil) in the Pacific Northwest (30), indicating that this method could be useful for disease forecasting and management. This sensitivity was higher than recent reports for *Meloidogyne* and *Nacobbus* spp., where the level of detection was 5,000 juveniles/kg of soil (14) and 30,000 juveniles/kg of soil (4), respectively.

During the present study, the presence of *P. neglectus* and *P. thornei* was successfully detected in most of the soil samples taken from Morrow County or Umatilla County, Oregon. Each pathogen was specifically detected in naturally infested field soils that contained a higher number of nematodes than the respective detection limits. Particularly, two soil samples were infested with both *P. neglectus* and *P. thornei* and, accordingly, the two species were detected by PCR. Compared with the Whitehead tray method and subsequent microscopic identification, the lack of *P. neglectus* DNA detection for S1, S2, and S3 or the lack of *P. thornei* DNA detection for S20 by PCR is probably due to the uneven distribution of nematodes in soil (23) and the small amount of soil (≤1 g) that can be processed in the FastPrep FP120 homogenizer. However, the Whitehead tray method requires a minimum of 48 h to extract approximately 60% of the nematode population from silt loams, and it may take as many as 6 days to extract all nematodes (5). In contrast, DNA extraction and PCR amplification, as used in this study, require 5 to 6 h.

Pratylenchus spp.-infested soil represents a serious threat to intolerant wheat cultivars. Therefore, the detection and discrimination of these nematodes in soil are very important in the Pacific Northwest. The molecular method developed in this study allows nematode DNA to be extracted directly from soil, facilitating the rapid and inexpensive detection and identification of *P. neglectus* and *P. thornei* with equipment commonly available in research and commercial laboratories. In commercial labs, *Pratylenchus* spp. are often quantified at the genus level, which is now recognized as inadequate for serving wheat-based agricultural systems where *Pratylenchus* spp.-specific tolerance and resistance are being developed. This PCR-based diagnostic method has the potential for implementation in commercial as well as research applications. Protocols reported here are being adapted for use in real-time PCR applications, thus enabling labs to avoid physical separation, microscopic identification, and counting of *P. neglectus* and *P. thornei* from soil.

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